

Evaluation of *Lolium temulentum* as a model grass species for the study of salinity stress by PCR-based subtractive suppression hybridization analysis

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Abstract

Soil salinity is one of the major abiotic stresses responsible for reduced persistence, yield and biomass accumulation in many crops including forage grass. Forage grass species are generally polymorphic, obligate out-crossers, that are self-incompatible. Because of their high genetic diversity, the mechanisms of salt tolerance are poorly understood. Consequently, the development of a useful model grass plant for the study of abiotic stresses is of great importance. We propose the use of *Lolium temulentum* L. (Darnel ryegrass), a diploid self-fertile species with a short life cycle (2–3 months), as a model system for the study of forage/turf grass species. To evaluate the utility of *L. temulentum* as a model grass species to study salt stress, a PCR-based subtractive suppression hybridization library was generated and sequenced. A total of 528 unique sequences were identified, among which 167 corresponded to orthologs of previously identified plant stress response genes. The expression patterns in leaf, crown and root tissues of selected genes were analyzed by Northern blot analysis, demonstrating salinity depended regulation of gene expression. These preliminary studies provide proof of concept supporting the use of *L. temulentum* as a model forage grass for molecular genetic analyses of salinity stress.

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1. Introduction

Over the past few decades, there has been a dramatic increase in the salinization of arable land [1,2]. As land becomes more limited for conventional agriculture, plants grown on marginal soils will be exposed to higher levels of soil salinity. Forage grasses are a critical component of feed used in livestock production worldwide, with many of these same species of grasses being utilized for, lawns, erosion prevention, and recreation. Soil salinity is one of the major abiotic stresses responsible for reduced persistence, yield and biomass accumulation in many crops, including forage. Consequently, it is of great importance to develop a better understanding of salt tolerance in forage species.

The consequences of salt stress can have a severe impact on the growth and development of the plant. Salt stress typically results in reduction in photosynthesis and nutrient uptake. Salt stress affects plants primarily through the creation of osmotic stress through the reduction of the water potential of the soil solution, and through the direct action of excess Na^+ and Cl^- ions [2–4]. In order to adapt to osmotic and Na^+ stress, plants have developed a variety of responses. Plants can rapidly induce genes involved in the production of compatible osmolites (osmoprotectants) to stabilize osmotic potential of its cells, to prevent dehydration and to ensure continued positive turgor pressure within cells. The action of these osmoprotectants improves water and nutrient uptake from the soil solution and helps ensure the continuation of vital plant processes [2–5]. In addition, to the synthesis of these osmoprotectants, plants respond to the toxic effect of Na^+ through the production of a host of ion transporters, such as Na^+ efflux transporters to stabilize ion concentrations within and

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outside plant cells [2–4]. Additionally, to reduce the toxic effects of reactive oxygen species (ROS) plants often increase their ROS scavenging potential through the up regulation of detoxification enzymes, such as superoxide dismutase (SOD) genes [3,4].

Such primary responses are often augmented by a host of other secondary responses, such as the re-enforcement of vital processes through the up regulation of genes involved in amino acid synthesis and carbon metabolism [3,4]. Furthermore, throughout the plant's response to salt stress, a variety of transcription factors, signaling proteins and compounds are used to regulate and coordinate expression of these genes and adaptations [2–4].

Over the past few years, considerable progress has been made towards understanding the mechanisms leading to salt tolerance in dicot species [1,2,4,6]. Unfortunately, much less progress has been made in the study of salinity tolerance in monocot species. Although homologs of genes known to be involved in salt tolerance have been identified in rice [7–9] and barley [10,11], very little is currently known on the molecular mechanisms used by forage grasses for salt tolerance.

The genetic intractability observed in many grass species, is primarily due to the fact that most are polyploid, self-incompatible, obligate out-crossers. Furthermore despite the phenotypic homogeneity displayed by many of these grasses, their populations possess a high degree of genetic heterogeneity. Where there can be as much genetic diversity among individual plants of the same variety as there is between varieties of the same species [12–15]. To further complicate matters, many forage grass species require a vernalization period to induce flowering. As such, the use of genetic approaches to study salt stress and tolerance can be impractical. Furthermore, many of these species are also not amenable to genetic transformation and/or tissue culture based regeneration systems, thereby limiting molecular approaches being utilized for evaluation of genes to be used for forage grass improvement. These problems clearly dictate the need for the development of a molecular model system for grass species.

Fortunately, *Lolium temulentum* (Darnel ryegrass) lacks many of the negative genetic attributes associated with most other grass species. *L. temulentum* is better suited for genetic and molecular genetic analyses than related grasses. Unlike other grasses, *L. temulentum* has a highly inbred diploid genome and has the capacity for self-fertilization. It is also a facultative long day plant that requires no vernalization for flowering [16,17]. *L. temulentum* also has a very short life cycle, which allows plants to grow to maturity in as little as 2–3 months, placing its development time on par with the model plant *Arabidopsis*. Furthermore *L. temulentum* has also been shown to be able to out-crossed with other *Lolium* species [18–20]. In addition, to these positive genetic attributes, *L. temulentum* shows great promise as a reverse genetic system. While transformation systems are still in development, this grass species is amenable to tissue culture and the subsequent regeneration [21]. Clearly, *L. temulentum* represents an ideal candidate for the continued development of a model grass system for use in plant research.

Therefore, we wanted to investigate *L. temulentum*'s utility for the molecular analysis of salt stress. Suppression subtractive hybridization (SSH) was used to identify differential expression of genes related to salt stress tolerance. The analysis of these genes demonstrated the utility of *L. temulentum* as a model grass system for the identification and isolation of genes up regulated in response to salt stress.

2. Materials and methods

2.1. Plant materials

Lolium temulentum L. cv. Ceres seeds were planted in approximately 1 L pots (4 in. × 4 in. × 4 in. pots) in SB40 Sunshine Growing Mix (Sun Gro Horticulture, Canada) or in two layers of vermiculite: fine (top) and coarse (bottom). Plants were fertilized weekly using Technigro 20-18-20 all-purpose fertilizer (Sun Gro Horticulture, Canada). Plants were grown in a Conviron E15 (Conviron, Winnipeg, Canada) growth chamber under 8 h photo-period at 21 °C day and 18 °C night.

2.2. Salt treatments

Subtraction library: plants were grown in soil for 6 weeks, then treated with 500 mL (s) of 500 mM NaCl, and incubated for 20 h. Leaf and crown tissue were collected, immediately frozen in liquid nitrogen and stored at –80 °C.

Northern analysis: plants were grown in vermiculite for 8 weeks, then treated with 500 mL (s) of 500 mM NaCl. Leaf, crown and root tissue were collected at 0, 12 and 24 h. Tissue was immediately frozen in liquid nitrogen and stored at –80 °C.

2.3. RNA isolation

Total RNA was isolated from harvested from leaves and crowns of salt treated (20 h) and non-treated tissues using the TRIzol reagent (Invitrogen, USA) according to the manufacturers' instructions. Poly(A+) RNA was isolated from 1 mg of the salt treated and non-treated total RNA with the Poly(A)Purist Kit (Ambion, USA). This mRNA was subsequently used for suppression subtractive hybridization.

2.4. Suppression subtractive hybridization

The subtraction library was prepared using PCR-Select cDNA subtraction kit according to the manufacturers' (Clonetech, CA) instructions. Tester and driver cDNAs were prepared from 2 µg of mRNA extracted from treated and non-treated tissues, respectively. The resulting PCR products were subsequently ligated into vector using the pGEM T Easy “TA” cloning kit (Promega, USA) using the manufacturer's recommendations.

2.5. Library preparation and sequencing

The ligation products of the SSH library were used to transform *E. coli* (TOPO10) competent cells (Invitrogen, USA).

Approximately, 768 well-separated white colonies were selected for sequencing. The library was maintained in 10×96 well plates, one replica set was shipped for sequencing to Rexagen Inc. (WA, USA). At Rexagen, DNA sequencing was performed with an automatic DNA sequencer (ABI prism, USA) using the T7 promoter primer.

2.6. Analysis of differentially expressed genes

The raw sequence information received from the sequencing facility was cleaned and trimmed of contaminating vector sequences using the Lucy package [22]. Sequences of low confidence (abundant N), extremely short (under 130 bp) or comprised of repetitive base composition were discarded. The remaining sequences were aligned and theoretical consensus sequences constructed using the Tigr Assembler 2 package [23]. The resulting unique sequences were then subjected to several BLAST, BLASTX [24] and RPS-BLAST [25] searches against GenBank and the CDD [26] databases to provide annotation information. The results of these searches were used to manually place the unique sequences into different categories base on proposed function.

2.7. Northern blot analysis of the transcripts

Gene candidates were selected based on annotation and frequencies and were subjected to further analysis of their expression patterns by Northern blot analysis. Leaf, crown or root tissue was ground to a powder using a mortar and pestle cooled with liquid nitrogen. Total RNA extractions from these ground tissues were performed using TRIzol reagent (Life Technologies, Gaithersburg, MD) following manufacturer instructions. Ten micrograms of total RNA was isolated from roots crowns and leaves of *L. temulentum* was electrophoretically separated on 1.2% denaturing formaldehyde agarose gels and blotted onto a Hybond N nylon membrane [27]. Inserts of selected cDNA were PCR-amplified using T7 and SP6 primers and a standard three-step protocol at 55 °C. The amplified fragments were then precipitated and rehydrated at a concentration of 25 ng/ μ L. 32 P-labeled DNA probes were generated from PCR-amplified fragments of selected cDNA, using the Ambion DECAprime II DNA labeling kit (Ambion, USA) and used to hybridize the RNA. The membranes were hybridized overnight with a 32 P-labeled probes (~100–200 cpm/mL) in a solution containing 50% formamide, 5× Denhart's solution, 0.1% (w/v) SDS, 6× SSPE, and 100 μ g/mL denatured salmon-sperm DNA at 42 °C. Filters were initially washed in 2× SSC and 0.1% SDS at 42–50 °C for 30 min, then subjected to a wash starting at 50 °C and allowing to cool to RT with shaking, and briefly in 0.2× SSC and 0.1% SDS at RT (°C) before autoradiography.

3. Results

To evaluate the utility of *L. temulentum* in the study of salt stress, mature, non-flowering plants were treated in soil with a solution of 500 mM NaCl or tap water. After 20 h symptoms of

osmotic stress were clearly visible on the treated plants, with slight loss in leaf stature and the initiation of leaf curling evident in the foliage of some tillers. The aerial portions of mature, non-flowering, *L. temulentum* plants (leaves and crowns) were harvested, and the salt induced expression pattern was investigated through the use of a PCR-based subtraction library. After subtraction, the resulting gene fragments were cloned and 768 random white colonies were selected and sequenced. After contiguous assembly, 528 unique sequences were identified with the average clone fragment size of 500–600 bp. The sequences were subjected to blast searches and the tentative annotation of each gene fragment was determined. Blast searches of the gene fragments against the protein database and the rice genome showed strong matches with many known genes.

The distribution of genes found in the library is shown in Table 1. The table has been categorized to highlight genes from 10 different functional categories based on our annotation. Blast searches of GenBank and protein motif scans using rpsblast (reverse position iterative blast) were performed for each gene and this information was used to determine their identity. Table 1 contains an ID number for each unique gene, the number of cloned sequences that were used to generate the contig or cluster and the final length of the gene fragment. Table 1 also includes the corresponding accession numbers for the top matches derived from searches of GenBank via blastx (six frame translation versus protein database) and blastn (nucleotide versus nucleotide) of the rice genome.

The analysis showed that genes commonly associated with plant stress dominated the profile. Over 30% of the sequences (167) detected in this library were in genes known to be associated with plant stress responses. Table 2 shows the final number of unique sequences found in each category. Collectively, the distribution of the genes detected appeared to match the patterns observed in other grass species, such as rice [9].

Nearly half of the stress related genes observed were metabolic enzymes responsible for the accumulation of compatible solutes in plant cells. We found 54 clones (comprised of 19 contigs) relating to proline synthesis (delta 1-pyrroline-5-carboxylate synthetase), 25 clones (comprised of 10 contigs) for glycine betane synthesis (aldehyde dehydrogenase) and 17 clones (comprised of 8 contigs) for sucrose synthase. Such genes are involved in reducing the initial impact of the osmotic stress. In addition, to these classic salt regulated genes, we also observed a number of cold and oxidative stress related genes, and genes involved in amino acid synthesis (glutamine-dependent asparagine synthetase) and lipid turnover (phospholipase D). The distribution pattern of genes up regulated in response to salt stress was consistent with the pattern of gene induction in other plant species [2,4,7–9].

It was interesting to note that many of the unique sequences appeared to form clusters corresponding to the same gene, as seen for the delta 1-pyrroline-5-carboxylate synthetase gene. Our analysis found that gene cluster 3 for the delta

Table 1

cDNA clones isolated from a subtractive hybridization library of salt stressed *Lolium temulentum* plants

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
Compatible solutes									
Cluster 1 ^a	25	698	Aldehyde dehydrogenase	AAG43027	289	7.0e−77	XP_472778	91	8.0e−19
Cluster 2 ^a	7	651	Beta-galactosidase	AAV59405	361	1.0e−98	XP_475792	361	1.0e−100
Cluster 3 ^a	54	862	Delta l-pyrroline-5-carboxylate synthetase	AAX35536	486	1.0e−136	NP_915492	414	1.0e−116
S7SLT_C387	1	367	Sucrose synthase 1	CAA04543	215	5.0e−55	NP_914696	145	6.0e−36
S7SLT_C159	1	114	Sucrose synthase 2	AAF85966	69	7.0e−11	NP_909830	66	4.0e−12
Cluster 4 ^a	15	775	Sucrose synthase 3	AAM89473	444	1.0e−123	NP_914696	348	2.0e−96
Detoxification									
S7SLT_C55	2	602	Peroxisome type ascorbate peroxidase	BAB62533	358	9.0e−98	XP_483666	342	1.0e−94
S7SLT_C312	1	696	Selenium-binding protein	XP_476776	320	4.0e−86	XP_476776	320	6.0e−88
S7SLT_C509	1	312	Catalase	CAH61266	44	1.0e−03	XP_463870	42	1.0e−04
Energy transfer									
Cluster 5 ^a	3	388	Alternative oxidase	BAB88645	169	2.0e−41	XP_473757	162	7.0e−41
S7SLT_C51	2	667	ATP-NAD kinase	AAX95220	197	2.0e−49	XP_470857	30	1.2e+00
S7SLT_C180	2	650	Electron transporter thiol-disulfide exchange protein	NP_172333	65	1.0e−09	XP_479574	63	1.0e−10
S7SLT_C102	1	473	NADH dehydrogenase 49kDa protein	CAA33911	119	3.0e−26	NP_039450	119	6.0e−28
S7SLT_C510	1	297	Plasma membrane H ⁺ ATPase	XP_476966	184	1.0e−45	XP_476966	184	1.0e−47
S7SLT_C19	1	694	Predicted mitochondrial carrier protein	AAV31266	160	3.0e−38	NP_912344	49	4.0e−06
S7SLT_C482	1	478	Vacuolar ATP synthase catalytic subunit A	Q40002	98	1.0e−19	XP_473481	31	3.9e−01
S7SLT_C342	1	590	Vacuolar H ⁺ -ATPase subunit B	BAA75517	327	1.0e−88	NP_916591	322	7.0e−89
S7SLT_C131	1	349	Vacuolar-type H ⁺ -translocating inorganic pyrophosphatase	BAD27918	145	5.0e−34	XP_476313	52	1.0e−07
General metabolism									
S7SLT_C168	1	741	1-Aminocyclopropane-l-carboxylate synthase	AAB18418	90	9.0e−17	XP_493697	84	6.0e−17
S7SLT_C398	1	326	12-Oxophytodienoate reductase	XP_482784	105	4.0e−22	XP_482784	105	5.0e−24
Cluster 6 ^a	2	673	Acid phosphatase	NP_849880	160	3.0e−38	NP_916580	97	8.0e−21
S7SLT_C154	5	186	Aconitase	AAAP30039	114	1.0e−24	XP_480473	113	3.0e−26
Cluster 7 ^a	5	601	Aconitate hydratase	XP_480473	389	1.0e−107	XP_480473	389	1.0e−109
S7SLT_C269	1	351	Acyl-CoA independent ceramide synthase	XP_469393	119	3.0e−26	XP_469393	119	3.0e−28
S7SLT_C479	1	498	Acyl-CoA oxidase ACX3	BAD35410	284	7.0e−76	XP_476282	86	1.0e−17
S7SLT_C128	2	374	Adenosylhomocysteinase-like protein	AAO72664	87	3.0e−16	XP_476137	27	4.1e+00
S7SLT_C309	1	709	ADP-ribosylation factor family	ABA94459	158	1.0e−37	XP_470055	99	3.0e−21
Cluster 8 ^a	3	619	Alanine-glyoxylate Aminotransferase	AAU44251	386	1.0e−106	XP_477982	92	3.0e−19
S7SLT_C491	1	436	Alpha-N-acetylglucosaminidase	CAAT7084	196	3.0e−49	XP_474139	164	1.0e−41
Cluster 9 ^a	2	446	Arabinoxylan arabinofuranohydrolase	AAK21880	308	3.0e−83	XP_471515	292	5.0e−80
S7SLT_C514	1	278	Asparagine synthetase	AAB71532	186	3.0e−46	XP_471842	27	3.7e+00
Cluster 10 ^a	3	571	Aspartate aminotransferase	XP_463436	355	6.0e−97	XP_463436	355	1.0e−98
S7SLT_C371	1	453	Auxin amidohydrolase	AAU06081	265	5.0e−70	NP_918395	254	2.0e−68
S7SLT_C261	2	377	Beta-adaptin protein A	NP_913231	241	5.0e−63	NP_913231	241	6.0e−65
Cluster 11 ^a	5	448	Branched-chain alpha keto-acid dehydrogenase	ABA95971	285	3.0e−76	XP_467697	69	1.0e−12
S7SLT_C348	1	578	Ceramide kinase	CAC39069	286	3.0e−76	XP_467121	284	3.0e−77
S7SLT_C200	1	617	Choline-phosphate cytidylyltransferase	XP_464309	88	2.0e−16	XP_464309	88	4.0e−18
Cluster 12 ^a	3	323	Cinnamoyl-CoA reductase	BAD35672	179	4.0e−44	XP_480400	104	1.0e−23
S7SLT_C47	2	615	Cinnamyl alcohol dehydrogenase	BAD73514	359	4.0e−98	NP_918057	359	1.0e−100
S7SLT_C179	1	663	CTP synthase	BAD68695	78	2.0e−13	NP_917309	78	4.0e−15
S7SLT_C458	1	600	Cytochrome P450	AAK38094	291	1.0e−77	NP_917788	258	1.0e−69
S7SLT_C190	4	630	Cytochrome P450 like_TBP	BAA10929	139	7.0e−32	XP_450634	30	8.6e−01
S7SLT_C220	1	565	Cytochrome P450-related protein	NP_909822	219	5.0e−56	NP_909822	219	1.0e−57
S7SLT_C215	1	581	Cytosolic aconitase	AAG28426	291	1.0e−77	XP_480473	290	5.0e−79
S7SLT_C212	1	590	D-Erythro-sphingosine kinase diacylglycerol kinase	NP_566064	137	2.0e−31	NP_921809	29	1.7e+00
S7SLT_C85	1	524	Dihydropyrimidine dehydrogenase	XP_467672	329	2.0e−89	XP_467672	329	5.0e−91
Cluster 13 ^a	2	539	Ethanolamine kinase 1	BAD36072	311	9.0e−84	NP_916653	125	2.0e−29

Table 1 (Continued)

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
S7SLT_C223	1	547	Ethylene-forming-enzyme-like dioxygenase-like p	XP_476311	33	6.6e+00	XP_476311	33	1.3e−01
S7SLT_C187	1	643	FadB 3-hydroxyacyl-CoA dehydrogenase	NP_908896	315	1.0e−84	NP_908896	315	2.0e−86
S7SLT_C119	1	424	Fumarylacetate hydrolase	XP_464472	189	3.0e−47	XP_464472	189	5.0e−49
S7SLT_C197	2	616	Galactose kinase	XP_470165	225	1.0e−57	XP_470165	225	2.0e−59
S7SLT_C242	1	473	Gamma-glutamyl cysteine synthetase	AAW58147	309	2.0e−83	XP_477983	301	1.0e−82
S7SLT_C314	2	679	GDP-mannose pyrophosphorylase	NP_915484	182	1.0e−44	NP_915484	182	2.0e−46
S7SLT_C207	1	599	Glucose-6-phosphate 1-dehydrogenase	NP_563844	246	3.0e−64	XP_477654	183	5.0e−47
S7SLT_C320	3	651	Glucose/sorbitone dehydrogenases	AAV49993	423	1.0e−117	XP_473336	100	6.0e−22
Cluster 14 ^a	5	465	Glutamine-dependent asparagine synthetase	AAU89392	273	2.0e−72	XP_476370	32	1.3e−01
S7SLT_C436	2	695	Glyceraldehyde 3-phosphate dehydrogenase	CAA42901	371	1.0e−101	XP_479895	358	1.0e−99
S7SLT_C108	2	457	Glycerol kinase	AAR88660	172	4.0e−42	XP_479688	29	1.0e+00
S7SLT_C99	1	486	Glycosyl hydrolase	XP_475552	110	2.0e−23	XP_475552	110	4.0e−25
Cluster 15 ^a	3	424	Glyoxysomal citrate synthase	BAD27711	282	3.0e−75	XP_464443	43	8.0e−05
S7SLT_C516	1	274	Homogentisate 12-dioxygenase	BAD67951	69	5.0e−11	NP_913355	29	7.5e−01
S7SLT_C494	1	407	Hydroxypyruvate reductase	XP_463779	158	5.0e−38	XP_463779	158	9.0e−40
S7SLT_C456	1	617	Isocitrate dehydrogenase	NP_912978	398	1.0e−110	NP_912978	398	1.0e−111
Cluster 16 ^a	3	286	Isovaleryl-CoA dehydrogenase	XP_475553	186	2.0e−46	XP_475553	186	3.0e−48
S7SLT_C390	1	354	Lipase	AAT77848	113	3.0e−24	NP_913160	95	1.0e−20
Cluster 17 ^a	2	416	Membrane bound O-acyltransferase-like	BAD29531	52	7.0e−06	XP_468296	26	6.9e+00
S7SLT_C416	1	222	Methylcrotonyl-CoA carboxylase beta chain	XP_482468	144	1.0e−33	XP_482468	144	1.0e−35
S7SLT_C488	1	447	Methylmalonate semialdehyde dehydrogenase	AAP15456	171	8.0e−42	XP_476941	167	3.0e−42
S7SLT_C441	1	672	Oxidoreductase protein	XP_469064	237	2.0e−61	XP_469064	237	4.0e−63
Cluster 18 ^a	5	613	Peroxisomal fatty acid beta-oxidation multifunctional protein	XP_464920	253	4.0e−66	XP_464920	253	8.0e−68
S7SLT_C231	1	512	Phosphatidylinositol kinase	XP_474277	231	1.0e−59	XP_474277	231	2.0e−61
S7SLT_C418	1	196	Phosphoinositide-specific phospholipase C	AAK01711	137	1.0e−31	XP_479620	137	1.0e−33
S7SLT_C394	1	337	Phospholipase	NP_922417	208	4.0e−53	NP_922417	208	5.0e−55
S7SLT_C54	5	936	Phospholipase D	BAA19467	354	3.0e−96	XP_470814	236	1.0e−62
S7SLT_C319	1	661	Phosphoribulokinase	CAB56544	246	6.0e−64	XP_467296	239	8.0e−64
S7SLT_C332	1	633	Predicted alpha/beta hydrolase	NP_174207	268	1.0e−70	XP_480055	121	3.0e−28
S7SLT_C71	4	554	Predicted hydrolase	AAZ15733	320	2.0e−86	NP_910889	306	7.0e−84
S7SLT_C445	1	648	Proteophosphoglycan	NP_922295	100	7.0e−20	NP_922295	100	1.0e−21
S7SLT_C96	1	501	Pyruvate dehydrogenase kinase 1	XP_479264	298	5.0e−80	XP_479264	298	1.0e−81
S7SLT_C16	1	709	S-Adenosylmethionine decarboxylase 2	XP_466676	281	1.0e−74	XP_466676	281	2.0e−76
S7SLT_C335	1	624	Serine hydroxymethyltransferase	ABA97575	371	1.0e−102	XP_475264	268	1.0e−72
S7SLT_C153	2	581	Succinic semialdehyde dehydrogenase	NP_178062	294	1.0e−78	XP_467607	133	6.0e−32
S7SLT_C344	1	589	Terpene synthase 7	AAS88577	209	6.0e−53	XP_471993	204	2.0e−53
S7SLT_C201	1	613	UDP-N-acetylglucosamine pyrophosphorylase	XP_480618	376	1.0e−103	XP_480618	376	1.0e−105
S7SLT_C277	1	307	UMP-CMP kinase	XP_468084	59	7.0e−08	XP_468084	59	9.0e−10
S7SLT_C409	1	279	Xylulose kinase-like	NP_912678	66	5.0e−10	NP_912678	66	6.0e−12
Membrane transport									
S7SLT_C61	1	589	ABC transporter	NP_915325	138	1.0e−31	NP_915325	138	3.0e−33
S7SLT_C316	2	666	ABC transporter ATP-binding protein	ABA94610	127	2.0e−28	NP_909539	37	8.0e−03
S7SLT_C439	1	692	Amino acid permease	ABA99423	53	8.0e−06	XP_469061	52	4.0e−07
S7SLT_C236	1	494	Auxin efflux carrier protein family	XP_477614	199	3.0e−50	XP_477614	199	7.0e−52
S7SLT_C232	1	510	Copper-transporting P-type ATPase	XP_464470	164	2.0e−39	XP_464470	164	3.0e−41
S7SLT_C275	1	329	MFS family transporter	NP_197538	160	2.0e−38	NP_913018	68	1.0e−12
Cluster 19 ^a	2	304	Mitochondrial phosphate transporter	BAA31583	193	1.0e−48	XP_462662	193	2.0e−50
S7SLT_C464	1	589	Multidrug-resistance associated protein	BAD69200	137	3.0e−31	NP_910489	137	6.0e−33
S7SLT_C288	1	223	Multidrug-resistance associated protein 1	AAO72316	72	5.0e−12	XP_476085	56	6.0e−09

Table 1 (Continued)

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
S7SLT_C523	1	197	Multidrug-resistance associated protein MRP1	AAL47686	49	6.0e−05	XP_474857	40	3.0e−04
S7SLT_C262	1	375	Na ⁺ H ⁺ antiporter	AAP93587	69	7.0e−11	XP_478236	27	2.4e+00
S7SLT_C404	1	301	PDR-type ABC transporter	BAD29207	189	3.0e−47	XP_482141	162	3.0e−41
S7SLT_C35	4	707	Predicted permease	AAT77085	182	9.0e−45	XP_482165	97	9.0e−21
S7SLT_C174	1	684	Sugar transporter protein	NP_922890	182	9.0e−45	NP_922890	182	2.0e−46
Plastid/mitochondrial genome									
S7SLT_C263	1	362	Chlorophyll A-binding protein CP47	CAA38541	223	1.0e−57	NP_039411	221	8.0e−59
S7SLT_C338	1	608	Chlorophyll AB-binding protein	BAD33211	89	8.0e−17	NP_916688	43	1.0e−04
S7SLT_C11	1	742	Chloroplast 50S ribosomal protein	Q95H50	134	3.0e−30	NP_039423	124	6.0e−29
S7SLT_C359	1	539	Chloroplast inner envelope protein 110 kDa	CAA92823	226	4.0e−58	NP_922115	225	1.0e−59
S7SLT_C107	2	458	Chloroplast predicted membrane protein	AAR91119	122	4.0e−27	NP_922308	28	3.0e+00
Cluster 20 ^a	5	641	Chloroplast ribosomal protein S8	NP_114293	267	2.0e−70	XP_481019	263	6.0e−71
S7SLT_C321	1	659	Cryptochrome Ia	XP_466372	207	3.0e−52	XP_466372	207	5.0e−54
S7SLT_C336	1	622	Methylcrotonyl-CoA carboxylase alpha chain	ABA99833	92	2.0e−17	XP_464438	29	2.4e+00
S7SLT_C504	1	361	Phosphoglucomutase precursor	XP_477902	206	2.0e−52	XP_477902	206	2.0e−54
S7SLT_C279	1	277	Photosystem II 10 kDa subunit	XP_476620	104	9.0e−22	XP_476620	104	1.0e−23
S7SLT_C79	3	538	PsaB	AAV74371	365	1.0e−100	NP_922434	365	1.0e−102
Protein degradation, folding and transport									
S7SLT_C323	1	655	26S proteasome RPT6a subunit	AAG42150	310	2.0e−83	XP_507461	305	1.0e−83
S7SLT_C349	1	575	Aminopeptidase N	XP_483801	359	3.0e−98	XP_483801	359	1.0e−100
Cluster 21 ^a	4	655	Aspartic proteinase	BAE20414	382	1.0e−105	XP_475576	368	1.0e−102
Cluster 22 ^a	10	682	ATP-dependent Clp protease ATP-binding subunit	AAN78327	312	7.0e−84	XP_466044	312	1.0e−85
S7SLT_C255	1	406	ATP-dependent LON1 protease	AAS19619	216	2.0e−55	NP_910416	113	4.0e−26
S7SLT_C217	1	573	Clp amino terminal domain	ABA96309	195	9.0e−49	XP_472335	193	6.0e−50
S7SLT_C473	1	523	Cysteine protease gp3b	AAW34137	76	6.0e−13	XP_475664	71	3.0e−13
S7SLT_C39	2	642	Cysteine proteinase Mir3	AAB88263	310	2.0e−83	XP_474131	303	4.0e−83
S7SLT_C15	1	722	Mitochondrial sorting protein (MSP1)	XP_479469	145	2.0e−33	XP_479469	145	3.0e−35
Cluster 23 ^a	3	591	Polyubiquitin	BAD61759	233	3.0e−60	XP_465025	202	9.0e−53
S7SLT_C181	1	655	Proteasome regulatory non-ATPase subunit	XP_470419	128	1.0e−28	XP_470419	128	2.0e−30
S7SLT_C266	2	353	Rab protein	CAA41850	45	1.0e−03	NP_917108	30	3.3e−01
S7SLT_C268	1	353	Serine carboxypeptidase I	ABA99132	120	1.0e−26	XP_467209	92	5.0e−20
S7SLT_C124	2	393	Serine carboxypeptidase I-related	AAX96495	172	3.0e−42	XP_467209	161	1.0e−40
S7SLT_C340	1	598	Serine carboxypeptidase III	P11515	387	1.0e−106	XP_463859	370	1.0e−103
S7SLT_C229	2	521	Ubiquitin-associated (UBA) protein	XP_466502	271	1.0e−71	XP_466502	271	2.0e−73
S7SLT_C247	1	450	Ubiquitin-conjugating enzyme E2	ABA99977	111	1.0e−23	NP_909850	109	7.0e−25
S7SLT_C196	1	626	Ubiquitin-specific protease 12	ABA98280	253	4.0e−66	XP_476711	207	4.0e−54
S7SLT_C270	1	350	UBX domain protein	AAX96152	151	7.0e−36	XP_483631	35	1.0e−02
S7SLT_C140	1	318	Zn-dependant endopeptidase	XP_478769	157	9.0e−38	XP_478769	157	1.0e−39
Signal transduction									
S7SLT_C182	2	659	14-3-3 protein	AAB33304	374	1.0e−102	XP_472763	374	1.0e−104
S7SLT_C59	1	599	Calnexin	CAA54678	159	6.0e−38	XP_472371	155	2.0e−38
S7SLT_C88	2	505	Calreticulin precursor	XP_470161	296	3.0e−79	XP_470161	296	6.0e−81
S7SLT_C208	1	598	Casein protein kinase 2 alpha subunit	BAD98469	359	3.0e−98	NP_919109	354	2.0e−98
S7SLT_C132	1	348	GTP-binding protein	XP_475372	112	6.0e−24	XP_475372	112	7.0e−26
S7SLT_C474	1	515	GTPase activating protein-like	BAD72476	274	1.0e−72	XP_467416	274	2.0e−74
S7SLT_C497	1	396	Kinase interacting protein	XP_468999	173	2.0e−42	XP_468999	173	2.0e−44
S7SLT_C27	1	679	Kinase-like protein splice variant 1	BAD73564	167	2.0e−40	NP_915999	167	4.0e−42
S7SLT_C101	1	473	Probable two-component sensor	AAG08367	32	5.8e+00	NP_914674	30	8.4e−01
Cluster 24 ^a	3	444	Protein kinase	XP_475843	274	7.0e−73	XP_475843	274	1.0e−74
S7SLT_C245	1	460	ATPase-like protein	XP_476929	224	8.0e−58	XP_476929	224	2.0e−59
S7SLT_C68	1	565	Auxin-regulated protein	XP_475977	305	4.0e−82	XP_475977	305	9.0e−84
S7SLT_C303	1	757	Beta-adaptin protein A	NP_192877	348	1.0e−94	NP_917777	110	7.0e−25
S7SLT_C308	1	710	Bromodomain-containing protein	XP_479904	111	3.0e−23	XP_479904	111	5.0e−25
S7SLT_C241	1	476	DMC1 DNA-binding protein	AAF42940	125	5.0e−28	XP_468126	33	7.7e−02
S7SLT_C22	1	688	DNA helicase	XP_467358	342	5.0e−93	XP_467358	342	9.0e−95

Table 1 (Continued)

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
S7SLT_C495	1	405	Protein kinase (PKC η) interacting protein	NP_922186	209	3.0e-53	NP_922186	209	6.0e-55
S7SLT_C81	1	538	Protein kinase SPK-3	NP_919612	150	3.0e-35	NP_919612	150	6.0e-37
S7SLT_C194	1	629	Protein kinase SPK-3	NP_915675	367	e-100	NP_915675	367	1.0e-102
S7SLT_C365	1	504	Protein phosphatase 2A regulatory subunit (Tonneau 2)	AAU44034	304	9.0e-82	XP_464419	63	1.0e-10
S7SLT_C337	1	616	Protein phosphatase 2A regulatory subunit A	AYY24685	259	3.0e-68	XP_450276	253	6.0e-68
S7SLT_C60	1	597	Protein phosphatase type 2C	AAM61437	76	9.0e-13	XP_472679	71	5.0e-13
S7SLT_C367	2	473	Protein phosphatase type 2C	BAD28017	301	4.0e-81	XP_473059	105	9.0e-24
S7SLT_C152	1	203	Receptor kinase (BRI1-KD) interacting protein	BAD11345	75	8.0e-13	XP_465592	29	5.7e-01
S7SLT_C91	1	511	Receptor protein kinase-like	BAD45621	139	4.0e-32	NP_919754	115	2.0e-26
S7SLT_C195	1	628	Receptor-protein kinase	NP_916295	212	9.0e-54	NP_916295	212	2.0e-55
S7SLT_C354	1	563	Rutative RGH1A	XP_480282	171	1.0e-41	XP_480282	171	3.0e-43
S7SLT_C451	1	634	Serine/threonine kinase	AAP82174	334	2.0e-90	XP_479600	310	6.0e-85
S7SLT_C100	2	473	Serine/threonine protein kinase	XP_472590	300	1.0e-80	XP_472590	300	3.0e-82
S7SLT_C99	1	486	Serine/threonine protein kinase	XP_475552	110	2.0e-23	XP_475552	110	4.0e-25
S7SLT_C246	1	458	Serine/threonine protein kinase	NP_922504	181	6.0e-45	NP_922504	181	1.0e-46
S7SLT_C167	1	742	Serine/threonine protein kinase (transmembrane)	BAD46417	296	6.0e-79	NP_911229	209	1.0e-54
S7SLT_C501	1	377	Serinethreonine protein phosphatase PP2A-1	BAD61854	100	2.0e-20	XP_464663	100	4.0e-22
S7SLT_C63	1	586	Small GTP-binding protein	AAW52512	325	6.0e-88	XP_466431	317	4.0e-87
Stress related									
S7SLT_C84	1	529	DnaJ domain containing protein	NP_922851	281	1.0e-74	NP_922851	281	2.0e-76
S7SLT_C476	1	506	Aluminum-induced protein (Wal17)	BAC78581	223	2.0e-57	XP_469697	223	5.0e-59
S7SLT_C448	2	635	Chloroplast drought-induced stress protein	XP_450158	142	1.0e-32	XP_450158	142	2.0e-34
S7SLT_C459	2	592	Cold acclimation protein WCOR413	AAL23724	188	1.0e-46	XP_469914	183	5.0e-47
S7SLT_C116	3	507	Dehydrin 2	AAS46614	39	5.9e-02	NP_917108	31	3.4e-01
S7SLT_C512	1	280	Dehydrin 7	AAX14224	55	6.0e-07	NP_917108	39	6.0e-04
S7SLT_C313	1	695	Dihydrolipoamide dehydrogenase	NP_908725	117	4.0e-25	NP_908725	117	6.0e-27
S7SLT_C310	1	698	DnaJ protein family	XP_483390	329	5.0e-89	XP_483390	329	8.0e-91
S7SLT_C318	2	657	HAL3B	BAD35576	343	4.0e-93	XP_471582	31	7.1e-01
S7SLT_C104	1	469	LEA D-II dehydrin	BAC01112	66	4.0e-10	NP_917108	39	2.0e-03
Cluster 25 ^a	23	669	Lysine ketoglutarate reductase/saccharopine dehydrogenase	AAG21985	309	6.0e-83	XP_468136	209	9.0e-55
Cluster 26 ^a	19	694	Saccharopine dehydrogenase	CAD48130	392	1.0e-108	XP_468135	328	1.0e-90
S7SLT_C406	2	293	Senescence-associated protein	BAB33421	189	2.0e-47	XP_463080	30	3.3e-01
S7SLT_C76	1	550	Senescence-related protein	BAA86060	101	1.0e-20	XP_467672	101	3.0e-22
Transcriptional factor									
S7SLT_C221	1	548	DNA-binding transcription factor	NP_190793	137	1.0e-31	XP_478750	39	2.0e-03
S7SLT_C225	1	541	DNA-binding protein GBP16	AAV44069	284	9.0e-76	XP_483588	70	7.0e-13
S7SLT_C53	1	611	Egalitarian-like	BAD45014	260	3.0e-68	NP_918466	228	3.0e-60
S7SLT_C21	1	691	ETTIN-like auxin response factor	AAQ86960	357	3.0e-97	NP_916153	337	4.0e-93
S7SLT_C57	2	594	F-box family protein	ABA91133	228	8.0e-59	XP_464514	49	3.0e-06
S7SLT_C31	1	663	F-box protein ORE9-like	BAD69289	156	6.0e-37	NP_917494	28	6.0e+00
S7SLT_C237	2	485	G-box binding factor	AAU10677	93	4.0e-18	NP_917345	70	5.0e-13
S7SLT_C175	1	682	G-box binding factor 8	NP_917345	229	6.0e-59	NP_917345	229	1.0e-60
S7SLT_C452	1	634	Initiation factor (iso)f p82 subunit	AAA74724	234	2.0e-60	XP_473052	200	5.0e-52
S7SLT_C114	1	436	LRR-containing F-box protein	AAU90110	259	3.0e-68	NP_915536	248	7.0e-67
S7SLT_C32	1	661	MybSt1	BAD72263	186	4.0e-46	NP_914529	186	8.0e-48
S7SLT_C33	2	660	Nucleotide-binding protein	NP_192866	231	2.0e-59	XP_479986	33	1.9e-01
S7SLT_C515	1	277	Replication factor C large subunit	CAC86668	166	3.0e-40	NP_912339	40	3.0e-04
S7SLT_C437	1	703	Response regulator 10	NP_912485	270	3.0e-71	NP_912485	270	6.0e-73
S7SLT_C253	2	405	RING-H2 finger protein	XP_483658	161	8.0e-39	XP_483658	161	1.0e-40
S7SLT_C209	1	595	SBP-domain	XP_470314	303	2.0e-81	XP_470314	303	4.0e-83
S7SLT_C97	2	491	SMAD6 interacting protein	BAD72492	235	5.0e-61	NP_917379	149	1.0e-36
S7SLT_C203	1	605	Transcription initiation factor	XP_474374	171	2.0e-41	XP_474374	171	4.0e-43
S7SLT_C283	1	269	Transcription regulatory protein	NP_922688	164	7.0e-40	NP_922688	164	9.0e-42
S7SLT_C13	1	735	Zinc finger protein	NP_915688	254	2.0e-66	NP_915688	254	4.0e-68
S7SLT_C206	1	599	Zinc finger protein	BAA33200	118	1.0e-25	NP_910981	75	4.0e-14

Table 1 (Continued)

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
Unclassified proteins									
S7SLT_C489	1	444	tRNA-binding protein	XP_471715	207	1.0e−52	XP_471715	207	2.0e−54
S7SLT_C249	1	449	AAA+ type ATPase	AAP80658	198	6.0e−50	NP_910388	150	2.0e−37
S7SLT_C438	1	692	Ankyrin kinase	BAD86971	149	1.0e−34	NP_916100	149	2.0e−36
S7SLT_C216	1	576	Ankyrin-like protein	BAD73402	323	2.0e−87	NP_915384	323	4.0e−89
S7SLT_C173	1	695	Arp2/3 complex-interacting protein VIP1/Aspl	AAT81732	384	1.0e−105	NP_916369	272	2.0e−73
S7SLT_C136	1	340	Asp-tRNAAsn/Glu-tRNAGln amidotransferase	XP_470957	221	7.0e−57	XP_470957	221	8.0e−59
S7SLT_C30	1	673	DNA topoisomerase II	XP_467311	154	3.0e−36	XP_467311	154	6.0e−38
S7SLT_C413	1	234	Endoplasmic reticulum membrane fusion protein	NP_921687	145	6.0e−34	NP_921687	145	7.0e−36
S7SLT_C395	2	331	Eukaryotic translation initiation factor	BAD30897	87	1.0e−16	XP_478659	27	3.7e+00
S7SLT_C410	1	270	Eukaryotic translation initiation factor 5A	AAG53645	85	7.0e−16	XP_469841	69	9.0e−13
S7SLT_C198	1	625	EXO70-G1 protein	BAD88371	174	3.0e−42	XP_465879	124	4.0e−29
S7SLT_C44	2	646	Glutamyl-tRNA synthetase	NP_912947	329	5.0e−89	NP_912947	329	9.0e−91
S7SLT_C443	1	657	Histone acetyltransferase (HAC13)	BAD37604	178	2.0e−43	XP_471330	30	9.1e−01
S7SLT_C428	1	755	Hydroxyproline-rich glycoprotein	BAD30888	161	2.0e−38	NP_913374	31	6.6e−01
S7SLT_C260	1	384	LEM3-like	BAD45383	138	7.0e−32	XP_475734	135	9.0e−33
S7SLT_C329	1	639	Meiosis protein Mei2	AAT39001	296	4.0e−79	XP_467506	97	8.0e−21
S7SLT_C188	5	837	MtN19	XP_483551	290	3.0e−77	XP_483551	290	6.0e−79
S7SLT_C287	1	231	Nuclear protein	BAD89467	98	9.0e−20	XP_483744	45	1.0e−05
S7SLT_C465	1	579	OSA15 protein	AAK59984	134	2.0e−30	XP_483532	28	4.7e+00
S7SLT_C28	1	678	Oxysterol-binding	NP_195830	352	5.0e−96	XP_469455	76	2.0e−14
S7SLT_C324	1	649	Pattern formation protein GNOM	XP_465291	285	9.0e−76	XP_465291	285	2.0e−77
S7SLT_C213	1	587	Pentatricopeptide (PPR) repeat-containing protein	XP_480482	341	8.0e−93	XP_480482	341	2.0e−94
S7SLT_C472	1	524	Plectin-related protein	BAD68172	121	1.0e−26	NP_915873	121	2.0e−28
S7SLT_C330	3	652	pp70 ribosomal protein S6 kinase	CAA56313	383	e−105	XP_479548	347	4.0e−96
S7SLT_C7	1	788	Pre-mRNA processing factor	AAV84869	153	7.0e−36	XP_470378	145	3.0e−35
S7SLT_C455	1	618	Prosaposin	ABA91163	237	2.0e−61	XP_550252	144	4.0e−35
S7SLT_C467	1	558	Ripening regulated protein	XP_482980	303	2.0e−81	XP_482980	303	4.0e−83
S7SLT_C118	1	425	SAP family cell cycle protein	XP_473921	100	1.0e−20	XP_473921	100	2.0e−22
S7SLT_C481	1	480	Selenoprotein O	BAD61584	126	3.0e−28	XP_465631	31	3.0e−01
S7SLT_C193	1	629	SNF7-like protein	XP_466538	150	2.0e−35	XP_466538	150	4.0e−37
S7SLT_C401	1	315	SPFH domain band 7 family	AAT85034	202	4.0e−51	XP_466631	31	2.0e−01
S7SLT_C38	4	640	Splicing factor	XP_636971	58	3.0e−07	XP_465115	48	5.0e−06
S7SLT_C352	1	572	Splicing factor	BAD28954	322	6.0e−87	XP_470874	80	6.0e−16
S7SLT_C46	1	621	Step II splicing factor SLU7	XP_479907	367	e−100	XP_479907	367	1.0e−102
S7SLT_C290	2	205	Thaumatin-like pathogenesis-related protein	P50696	101	1.0e−20	XP_469149	39	7.0e−04
S7SLT_C525	1	174	tRNA synthetase	AAN08648	59	6.0e−08	NP_921080	59	7.0e−10
S7SLT_C23	1	687	tRNA-dihydrouridine synthase	XP_462698	250	3.0e−65	XP_462698	250	6.0e−67
S7SLT_C52	1	614	Wax biosynthesis regulator (eceriferum3)	CAB85536	83	7.0e−15	XP_483424	27	8.9e+00
S7SLT_C42	2	629	Unidentified homologous EST only	XP_463448	283	3.0e−75	XP_463448	283	6.0e−77
S7SLT_C37	2	639	Unidentified homologous EST only	XP_482395	49	1.0e−04	XP_482395	49	2.0e−06
S7SLT_C92	2	652	Unidentified homologous EST only	BAD29318	270	3.0e−71	NP_919524	28	3.5e+00
S7SLT_C83	2	524	Unidentified homologous EST only	AAU10753	174	1.0e−42	XP_469578	28	2.2e+00
S7SLT_C80	2	666	Unidentified homologous EST only	NP_922355	126	5.0e−28	NP_922355	126	1.0e−29
S7SLT_C369	1	461	Unidentified homologous EST only	BAD88119	119	4.0e−26	XP_475495	103	4.0e−23
S7SLT_C122	1	401	Unidentified homologous EST only	BAD72343	215	5.0e−55	NP_916786	215	7.0e−57
S7SLT_C356	1	553	Unidentified homologous EST only	CAB62634	127	2.0e−28	XP_463545	66	1.0e−11
S7SLT_C8	1	787	Unidentified homologous EST only	AAV49993	151	3.0e−35	XP_473336	47	1.0e−05
S7SLT_C393	1	341	Unidentified homologous EST only	XP_482599	211	9.0e−54	XP_482599	211	1.0e−55
S7SLT_C70	1	563	Unidentified homologous EST only	NP_908807	221	8.0e−57	NP_908807	221	2.0e−58
S7SLT_C265	1	359	Unidentified homologous EST only	BAD37521	92	6.0e−18	XP_464385	87	3.0e−18
S7SLT_C388	1	362	Unidentified homologous EST only	ABA93506	177	1.0e−43	XP_469567	47	3.0e−06
S7SLT_C106	1	465	Unidentified homologous EST only	XP_479102	174	1.0e−42	XP_479102	174	3.0e−44
S7SLT_C183	1	650	Unidentified homologous EST only	BAD68206	254	2.0e−66	NP_915942	254	4.0e−68
S7SLT_C391	1	345	Unidentified homologous EST only	NP_919108	107	2.0e−22	NP_919108	107	2.0e−24

Table 1 (Continued)

Sequence ID	Clones contained	Length	Annotation	Top blastx alignment			Top rice genome alignment		
				Accession	Score	Value	Accession	Score	Value
S7SLT_C307	1	712	Unidentified homologous EST only	XP_483534	211	1.0e-53	XP_483534	211	3.0e-55
S7SLT_C334	1	631	Unidentified homologous EST only	XP_483307	157	2.0e-37	XP_483307	157	4.0e-39
S7SLT_C346	1	584	Unidentified homologous EST only	XP_506633	208	8.0e-53	XP_506633	208	2.0e-54
S7SLT_C454	1	618	Unidentified homologous EST only	XP_467483	250	3.0e-65	XP_467483	250	5.0e-67
S7SLT_C471	1	534	Unidentified homologous EST only	NP_915272	242	4.0e-63	NP_915272	242	8.0e-65
S7SLT_C325	1	643	Unidentified homologous EST only	XP_469567	197	3.0e-49	XP_469567	197	5.0e-51
S7SLT_C506	1	349	Unidentified homologous EST only	ABA96799	146	2.0e-34	XP_466656	107	2.0e-24
S7SLT_C248	1	450	Unidentified homologous EST only	XP_477275	169	3.0e-41	XP_477275	169	6.0e-43
S7SLT_C327	1	641	Unidentified homologous EST only	XP_474331	150	4.0e-35	XP_474331	150	8.0e-37
S7SLT_C282	1	272	Unidentified homologous EST only	XP_550419	81	1.0e-14	XP_550419	81	2.0e-16
S7SLT_C165	1	111	Unidentified homologous EST only	XP_469638	218	2.0e-55	XP_469638	218	3.0e-57

For the sake of brevity an 188 unique clones with no known annotation have been omitted, please see supplementary data.

^a Clusters of genes with the same annotation have been compressed into single groups, please see supplementary data for individual sequence information.

1-pyrroline-5-carboxylate synthetase gene contained 19 different sequence designations or contigs (data not shown—please see online supplementary data set). One explanation for this result is that different sections of the same gene formed multiple contigs, due to gaps caused by missing sequence information. This is a direct result of how the SSH libraries are constructed, where the average fragment size in the library is roughly 500–600 bp, and therefore insufficient for whole EST spanning. Additionally, the conservative parameters used to assemble the contigs prevented sequences from becoming merged in a single contiguous sequence despite there being as much as 95% (in some cases) identity to one another. Conversely, poorly matching sequences may represent multiple isoforms or family members of the same gene.

In addition, to genes that have probable roles in stress responses, 123 clones relating to general metabolism were observed. Genes, such as, glyoxysomal citrate synthase and glutamine-dependent asparagine synthetase, demonstrate the probable need for additional re-enforcement of critical metabolic pathways under salt stress. In addition, to critical processes, such as glycolysis, there were a number of genes that had no clear relation to salt stress, genes such as branched-chain alpha keto-acid dehydrogenase or isovaleryl-CoA dehydrogenase, which may be involved in the stress response, however,

their roles are not well defined. Furthermore, our analysis found that over 40% (212 clones) of the total sequenced clones corresponded to novel gene fragments that had no previously identified function that could be determined by this researcher, despite matches to other EST and genomic sequences in GenBank. Consequently, the specific annotation of these sequences could not be readily determined, and for the sake of brevity many of these sequences/clones were omitted from Table 1. However, it is interesting to speculate that these “unknown” yet expressed gene candidates, could play an important role in the salt stress response. The entire collection of sequences were submitted to GenBank and are represented by accession numbers EB709553-EB710082.

In order to determine the validity of the library with respect to salt induced expression, selected genes from different categories of the library were analyzed by Northern blot analysis. *L. temulentum* plants were subjected to 0, 12 or 24 h of salt stress and total RNA was extracted from root, crowns and leaves. The delta 1-pyrroline-5-carboxylate synthetase [28] and aldehyde dehydrogenase [29] genes have previously been shown to be involved in the biosynthesis of compatible solutes in response to salt stress in other plant species. While the cold acclimation protein WCOR413, phospholipase D and small GTP-binding protein genes have less defined expression patterns as they relate to salt stress. In addition, to the five selected genes from the library, the polyubiquitin gene isolated from *L. temulentum* (data not shown) was included as control. As shown in Fig. 1, delta 1-pyrroline-5-carboxylate synthetase and aldehyde dehydrogenase genes were strongly induced in crown and leaf tissue 12 h after exposure to salt stress. All of the genes except for the delta 1-pyrroline-5-carboxylate synthetase, displayed a transient induction, with the highest transcript level appearing after 12 h of salt stress in most tissues tested. Surprisingly, we observed differential gene expression in root tissue even though this tissue was not used in construction of the library. Indicating the utility of these genes to be used to dissect temporal and tissue specific responses in the plant. Furthermore, the phospholipase D and small GTP-binding protein genes displayed low level constitutive expression in all tissue prior to salt stress. After exposure to salt stress these genes showed increase transcript accumulation in all tissues after

Table 2
Distribution of clones by functional category

Category	Total sequences
Compatible solutes	103
Detoxification	4
Energy transfer	13
General metabolism	123
Membrane transport	19
Plastid/mitochondrial genome	18
Protein degradation, folding and transport	38
Signal transduction	34
Stress related	60
Transcriptional factor	26
Unclassified proteins	88
Unidentified proteins	212
Unique sequences	528
Total clones sequenced	738

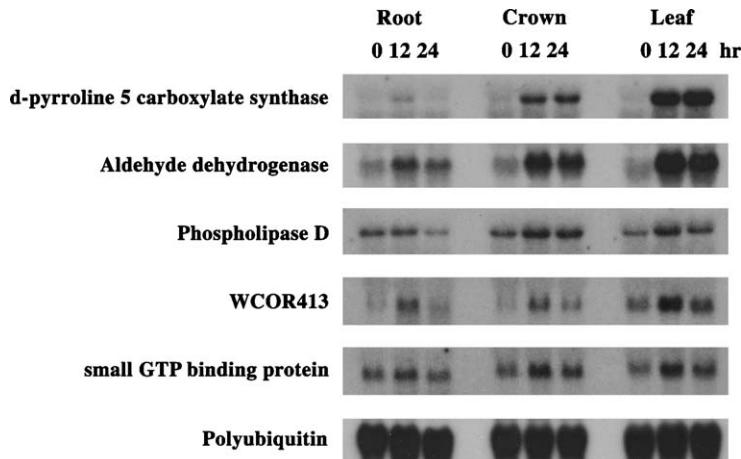


Fig. 1. Northern blot analysis of salt induced expression from selected genes isolated from the subtractive library from *L. temulentum* plants. Delta 1-pyrroline-5-carboxylate synthetase (S7SLT-C36); aldehyde dehydrogenase (S7SLT-C17); phospholipase D (S7SLT-C54); WCOR 413 (S7SLT-C459); small GTP-binding protein (S7SLT-C63) and one constitutive control, Polyubiquiton to show equal loading: total RNA was extracted from root, crown and leaf tissue from plants that had been treated with 500 mM NaCl for the indicated number of hours (0, 12 or 24 h). Each lane was loaded with 10 µg total RNA and run on an agarose gel. Equal loading of RNA per lane was confirmed by visualization with ethidium bromide of the gel before blotting and the nylon membrane after blotting (data not shown).

12 h, except for the phospholipase D gene. This gene showed characteristic accumulation of message in shoots after 12 h. However, in root tissue the phospholipase D gene demonstrated no increase in transcript levels after 12 h and ultimately was down regulated after 24 h in root tissues. The control polyubiquitin probe exhibited nearly identical expression in all treatments, indicating even loading and unbiased expression profiles.

4. Discussion

The primary focus of this research was the identification of genes that are up regulated in *L. temulentum* under salt stress. The results obtained using the SSH approach demonstrated the benefits of this model grass, yielding a high-resolution profile of genes induced by salt stress. The cDNA subtraction strategy identified over 500 unique gene candidates for further study. Blast searches of these genes showed that many of isolated genes had significant homology to sequences in GenBank, and were likely involved in plant stress responses. These genes collectively play a role in detoxification, biosynthesis of osmoprotectants and the maintenance of critical cellular metabolic processes [2–4]. The distribution and identity of these genes were consistent with the expression patterns observed in other plant species under salt stress.

Northern blot analysis of selected genes showed clear salt regulated expression in different plant tissues. It was encouraging to find that even novel and uncharacterized genes such as the small GTP-binding protein showed salinity regulated expression (Fig. 1). It is tempting to envision that some of the genes identified in Table 1, associated with signal transduction, such as the GTP-binding protein, or putative transcription factors play an important regulatory role in salt stress responses in grasses. It will be interesting to investigate the expression patterns and roles played by many of the other novel or unknown genes identified by this method.

Due to the limited research tools available in the study of forage and turf grasses, the development of model systems is of paramount importance. *L. temulentum* with its short generation time, diploid genome and self-fertile nature appears to be an ideal candidate for future application of large scale studies utilizing modern molecular tools. This grass species has been shown to be amenable to tissue culture, plant breeding and gene expression profiling as demonstrated by this study. The results describe herein, demonstrated that the SSH approach provided valuable insight into the expression patterns of genes associated with salt stress in this model grass species. Our analysis detected a number of genes commonly associated with salt stress in addition to several novel transcripts. It is becoming clear that despite the wealth of information and work in this field, there still remain many unknown components in the plant's response to salt stress. One of the first steps towards developing a better understanding of stress responses is the careful selection of model plants, and the detailed profiling of the genes involved in the stress response. Ultimately, the identification of stress tolerance genes and their subsequent characterization will lead to molecular and genetic approaches for forage and turf grass improvement.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2006.05.003.

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